Dietary Supplement 4-Methylumbelliferone: An Effective Chemopreventive and Therapeutic Agent for Prostate Cancer

Travis J. Yates, Luis E. Lopez, Soum D. Lokeshwar, Nicolas Ortiz, Georgios Kallifatidis, Andre Jordan, Kelly Hoye, Norman Altman, Vinata B. Lokeshwar

Affiliations of authors: Sheila and David Fuente Graduate Program in Cancer Biology, Sylvester Comprehensive Cancer Center (TJY, AJ, KH), Department of Urology (LEL, NO, GK, VBL), Honors Program in Medical Education (SDL), Department of Pathology (NA), Department of Cell Biology (VBL), Clinical Translational Science Institute (VBL), University of Miami-Miller School of Medicine, Miami, FL.

Current affiliation: Department of Cancer Biology, University of Pennsylvania, Philadelphia, PA (TJY).

Correspondence to: Vinata B. Lokeshwar, PhD, Department of Urology (M-800), Miller School of Medicine, University of Miami, P.O. Box 016960, Miami, FL 33101 (e-mail: vlokeshw@med.miami.edu).

Abstract

Background: Prevention and treatment of advanced prostate cancer (PCa) by a nontoxic agent can improve outcome, while maintaining quality of life. 4-methylumbelliferone (4-MU) is a dietary supplement that inhibits hyaluronic acid (HA) synthesis. We evaluated the chemopreventive and therapeutic efficacy and mechanism of action of 4-MU.

Methods: TRAMP mice (7–28 per group) were gavaged with 4-MU (450 mg/kg/day) in a stage-specific treatment design (8–28, 12–28, 22–28 weeks). Efficacy of 4-MU (200–450 mg/kg/day) was also evaluated in the PC3-ML/Luc+ intracardiac injection and DU145 subcutaneous models. PCa cells and tissues were analyzed for HA and Phosphoinositide 3-kinase (PI-3K)/Akt signaling and apoptosis effectors. HA add-back and myristoylated Akt (mAkt) overexpression studies evaluated the mechanism of action of 4-MU. Data were analyzed with one-way analysis of variance and unpaired t test or Tukey’s multiple comparison test. All statistical tests were two-sided.

Results: While vehicle-treated transgenic adenocarcinoma of the prostate (TRAMP) mice developed prostate tumors and metastases at 28 weeks, both were abrogated in treatment groups, without serum/organ toxicity or weight loss; no tumors developed at one year, even after stopping the treatment at 28 weeks. 4-MU did not alter the transgene or neuroendocrine marker expression but downregulated HA levels. However, 4-MU decreased microvessel density and proliferative index (P < .0001). 4-MU completely prevented/inhibited skeletal metastasis in the PC3-ML/Luc+ model and DU145-tumor growth (85–90% inhibition, P = .002). 4-MU also statistically significantly downregulated HA receptors, PI-3K/CD44 complex and activity, Akt signaling, and β-catenin levels/activation, but upregulated GSK-3 function, E-cadherin, and apoptosis effectors (P < .001); HA addition or mAkt overexpression rescued these effects.

Conclusion: 4-MU is an effective nontoxic, oral chemopreventive, and therapeutic agent that targets PCa development, growth, and metastasis by abrogating HA signaling.

Effective control of localized prostate cancer (PCa) and of its metastatic spread by consumption of a nontoxic dietary supplement can potentially delay/avoid treatment of low-risk localized PCa and halt progression in patients with advanced disease. 4-methylumbelliferone (4-MU; 7-hydroxy–4-methylcoumarin or hymecromone) is a dietary supplement consumed in Europe...
Supplementary also investigated the mechanism of action of 4-MU. Skeletal metastasis, and DU145 subcutaneous implantation. We have previously reported that, at the IC50, of 4-MU to inhibit HA synthesis is approximately 0.4 mM (~70 μM; [28–30]). 4-MU also downregulates the expression of HAS2, HAS3, and UDP-dehydrogenase; these are key enzymes in glycosaminoglycan synthesis (31,32).

Because of its fluorescence, 4-MU is widely used as a fluorescent indicator in enzyme assays. In small clinical trials, 4-MU has shown cholestatic and antispasmodic properties with improvement in liver and gallbladder functions (14,33–35). Although a coumarin-derivative, 4-MU lacks antisperminogenic and anti-atheromatous activities of coumarin, and unlike Coumadin it has no anticoagulant activity (36–39). The maximum tolerated dose of 4-MU in mice is 2.8 to 7.3 g/kg [National Institute for Occupational Safety and Health (NIOSH) registry: registry of toxic effects of chemical substances (RTECs) number GN7000000]. We have previously reported that, at the IC50 for HA synthesis, 4-MU inhibits proliferation, invasion, and motility of PCa cells in vitro and subcutaneous growth of PC3-ML xenografts (39). 4-MU has also shown antitumor activity in a few tumor models at doses of 1 to 3 g/day; however, 4-MU has not been evaluated for its chemopreventive activity and/or therapeutic efficacy in transgenic models, at various stages of cancer progression, or at doses comparable with those in human use [23–25,27]. We evaluated chemopreventive and therapeutic efficacy of 4-MU in three Pca mouse models—TRAMP, PC3-ML/Luc, and DU145 subcutaneous implantation. We also investigated the mechanism of action of 4-MU.

### Methods

#### Cells and Reagents

Culture conditions for human PCA cell lines DU145, PC3-ML, and LNCaP cells are described in the Supplementary Methods (available online). Sodium salt of 4-MU (MW: 198) was purchased from Sigma-Aldrich, Saint Louis, MO; other reagents and antibodies are listed in the Supplementary Methods (available online).

#### TRAMP Model

TRAMP-positive (T+) mice from each breeding (Supplementary Methods, available online) were randomly divided into vehicle and treatment groups. Mice in the vehicle group (n = 28) were orally gavaged daily with 2% sucrose starting at eight weeks and were killed at 22, 28, or 35 weeks. In the treatment groups, mice were gavaged with 450 mg/kg of 4-MU starting at eight (n = 15), 12 (n = 7), or 22 (n = 8) weeks of age for up to 28 weeks. In all treatment groups, 50% of mice were killed at 28 weeks, and the remaining 50% were observed without treatment for up to 52 (8–28 week group), 44 (12–28 week group), or 34 (22–28 week group) weeks. Because the mice in the vehicle group developed large prostate (P) tumors that invaded and fused with seminal vesicles (SVs), the weights of P+SV were measured together. Organ histology was evaluated by a board-certified veterinary pathologist (NA); immunohistochemistry is described in the Supplementary Methods (available online).

#### Intracardiac Skeletal Metastasis Model

PC3-ML/Luc+ cells (5x10⁴ cells/0.1 cc) were injected in the left ventricle of seven- to eight-week-old male athymic mice under anesthesia (Ketamine [80 mg/kg] + Xylazine [10 mg/kg] i.m. injection). Mice (six per group) were gavaged daily with 2% sucrose (vehicle) or 4-MU. 4-MU treatment started five days before tumor cell injection (Group 1: 200 mg/kg; Group 2: 450 mg/kg) or on the day of tumor cell injection (Group 3: 450 mg/kg). Mice were imaged weekly using IVIS live animal imaging. Mice in the vehicle group were killed after seven weeks. In treatment groups, the treatment was stopped at seven weeks but the imaging continued until week 14.

#### Subcutaneous Xenograft Model

DU145 cells (2x10⁴ cells) were injected with 50% Matrigel (BD Biosciences; San Jose, CA) on the dorsal flank of seven- to eight-week-old male athymic mice (seven per group). Mice were treated with vehicle or 4-MU (450 mg/kg) starting on day 0 or on day 8, when tumors became palpable. Tumor volume was measured twice weekly until day 28. On day 28, the treatment in the day 0 group was suspended and tumor growth was monitored until day 46.

#### 4-MU, HA Treatments, and Transient Transfections

PC3-ML and DU145 cells were incubated with 4-MU (0–0.6 mM) and/or HA (50 μg/mL) for 48 hours. Alternatively, PC3-ML cells were transiently transfected with a pcDNA3-Myr-HA-Akt1 plasmid using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Twenty-four hours following transfection, myristoylated Akt (mAkt)–expressing cells were treated with 4-MU (0.4 mM) for 48 hours. PC3-ML cells were also transfected with ON-TARGET plus CD44 and/or RHAMM siRNAs or control nontargeting siRNA. Following various transfections and treatments, cells were analyzed for transcript and protein expression by RT-q-PCR and immunoblotting, respectively (Supplementary Methods and Supplementary Table 1, available online) (10,40). PC3-ML cells treated with 4-MU (0 or 0.4 mM) for 24 hours were subjected to
phosphoinositide 3-kinase (PI-3K) activity assay using a PI-3K p85 colorimetric ELISA kit (Active Motif, Carlsbad, CA) or to proximal ligation assay (PLA; using anti-PI-3K and CD44 antibodies) (Supplementary Methods, available online) (41).

Statistical Analysis

Mean and standard deviation (SD) were computed for quantifiable parameters (e.g., P+SV weight, microvessel density [MVD], proliferation index, and transcript levels). Differences among the mean responses between different groups and tumor growth (DU145 model) were compared by one-way analysis of variance (ANOVA) followed by either unpaired t test (e.g., vehicle vs. 8–28 week group) or Tukey’s multiple comparison test when comparing more than two groups (e.g., control, HA, 4-MU, HA+4-MU). Statistical analyses were performed using the GraphPad Prism software, version 4.03. All statistical tests were two-sided.

Results

Efficacy of 4-MU in the TRAMP Model

The TRAMP model progresses through the following stages: normal, hyperplasia, prostatic intra-epithelial neoplasia (PIN: 6–8 weeks), well-differentiated adenocarcinoma (8–14 weeks), poorly differentiated adenocarcinoma (14–22 weeks), and metastasis (22–28 weeks) (42,43). Since we have reported potent antitumor activity of 4-MU (450 mg/kg) in a PCA xenograft model (39), we evaluated the efficacy of 4-MU (450 mg/kg) in the TRAMP model using a stage-specific treatment design. This dose is six to 16 times lower than the reported maximum tolerated dose for 4-MU in the NIOSH registry. Because of tumor burden, P+SV weights in 22- and 28-week-old vehicle-treated T+ mice (mean, SD = 0.9 g, 0.043 and 2.6 g, 2.5, respectively) were statistically significantly higher than the weights in 28-week-old TRAMP-negative (T-neg) mice (mean, SD = 0.46 g, 0.055; P < .0001) (Figure 1A). However, P+SV weights in the 8 to 28 and 12 to 28 (mean, SD = 0.48 g, 0.28 and 0.35 g, 0.035, respectively) week treatment groups were similar to those in T-neg mice (P > .8), suggesting that 4-MU both prevented and inhibited PCA growth. P+SV weights in the 22 to 28 week treatment group (mean, SD = 1.1 g, 0.35) were similar to those in 22-week-old T+ mice (P = .28), suggesting that even at late stages 4-MU inhibited tumor growth (Figure 1A). Furthermore, no serum toxicity or weight loss was observed in any treatment groups (Supplementary Figure 1A and Supplementary Table 2, available online).

Prostate specimens from 28-week-old vehicle-treated mice showed histological features consistent with a poorly differentiated, angiogenic invasive adenocarcinoma with tumor extending into SV (Figure 1B). Contrarily, all specimens in the 8 to 28 and 12 to 28 week treatment groups showed normal areas of prostate and prostate acini with low-grade PIN, a characteristic of the eight-week-old T+ prostate (Figure 1B). While the 22-week-old T+ prostate tissues showed morphological features of a

Figure 1. Therapeutic efficacy of 4-methylumbelliferone (4-MU) in the transgenic adenocarcinoma of the prostate (TRAMP) model. A) Weights of prostate and seminal vesicles (P+SV) in T-negative (T-neg) mice (n = 4) at 28 weeks and in TRAMP-positive (T+) mice at 22 (n = 4) and 28 weeks (n = 17). Data are means, and error bars represent standard deviation. B) Hematoxylin and eosin staining of prostate specimens from T- and T+ mice at 8, 12, and 22 weeks of age and from the vehicle and treatment groups at 28 weeks of age. Scale bar = 400X magnification. C) Kidney, liver, and lung specimens from T-neg and T+ (vehicle and 4-MU treatment groups) mice at 28 weeks of age are shown. Arrows indicate metastatic foci in specimens from the vehicle groups. 400X magnification. D) Prostate specimens from the vehicle and treatment groups were subjected to hyaluronic acid (HA), Ki67, CD34, and E-cadherin staining using immunohistochemistry. 400X magnification. 4-MU = 4-Methylumbelliferone.
poorly differentiated carcinoma present in fused acini, the 22 to 28 week treatment group showed high-grade PIN, hyperplastic regions, and intact prostate acini (Figure 1B). At 28 weeks, while 60% of the vehicle-treated animals were positive for metastases in the kidney, liver, and/or lung, none in the treatment groups showed organ metastasis (Figure 1C; Supplementary Table 3, available online). The organ histology in the 4-MU-treated and T-neg mice was the same, suggesting that long-term 4-MU treatment did not cause organ toxicity (Figure 1C).

**HA Expression and Related Phenotypes in TRAMP Tissues**

Consistent with 4-MU being an HA synthesis inhibitor, while prostate tissues from the vehicle-treated animals showed strong HA expression (ie, 3+ staining) in tumor cells and tumor-associated stroma, only weak HA expression (ie, 0 to 1+ staining) was observed in all treatment groups (Figure 1D). Proliferation indices were statistically significantly reduced in treatment groups (mean, SD = 8–28 wks: 1.6, 1.9; 12–28 wks: 1.2, 0.8; 22–28 wks: 2.8, 2.0) when compared with the vehicle group (50.8, 7.5; P < .0001) (Figures 1D and 2A). Similarly, MVD was also statistically significantly reduced in the treatment groups (mean, SD = 8–28 wks: 1.2, 1.3; 12–28 wks: 1.2, 0.8; 22–28 wks: 2.8, 2.4, 1.7) when compared with the vehicle group (20.8, 7.4; P < .0001) (Figures 1D and 2B). While there was a complete loss of E-cadherin expression in the prostate specimens from vehicle-treated animals, E-cadherin was strongly expressed in all treatment groups (Figure 1D).

In the TRAMP model, under the control of an androgen-responsive minimal probasin promoter, the transgene SV40 T-antigen initiates tumorigenesis in the prostatic epithelium. However, 4-MU did not downregulate SV40 T-antigen expression in any treatment group (Supplementary Figure 1B, available online). In advanced stages, the TRAMP model displays neuroendocrine phenotype (44,45). When compared with T-neg mice prostate tissues from 28-week-old T+ mice showed a statistically significant increase in the expression of neuroendocrine markers, chromogranin A (mean, SD = T+: 1.7, 0.36; T-neg: 0.02, 0.006), Foxa1 (T+: 6.5, 1.4; T-neg: 0.27, 0.11), Foxa2 (T+: 2.1, 0.34; T-neg: 0.13, 0.04), and synaptophysin (T+: 7.4, 0.3; T-neg: 0.11, 0.004). However, even the longest duration of 4-MU treatment (8–28 week) did not appreciably decrease the levels of these markers in the prostate tissues of treated animals (Supplementary Figure 1C, available online).

**Prevention of PCa in the TRAMP Model**

Although 4-MU treatment was terminated after 28 weeks, mice in the 8 to 28, 12 to 28, and 22 to 28 week groups did not develop prostate tumors even after 52, 44, and 34 weeks, respectively (Figure 2C). Furthermore, except for one mouse in the 22 to 28 week group, which developed metastasis after 34 weeks, no other animals in any treatment group developed metastasis (Supplementary Table 3, available online).

**Effect of 4-MU on HA Signaling and Epithelial Mesenchymal Transition**

The hallmark of epithelial mesenchymal transition (EMT) is upregulation and activation of β-catenin and subsequent upregulation of transcription factors Zeb1, Zeb2, Twist, and Snail via the LEF/TCF complex; this, in turn, negatively regulates E-cadherin expression (46,47). Post-translationally, β-catenin activation/levels are regulated by Akt and glycogen synthase-3 (GSK-3) α/β (48,49). While direct Akt phosphorylation of β-catenin at Ser552 induces its nuclear translocation, phosphorylation at Thr41/Ser45 by GSK-3 α/β marks it for degradation. GSK-3 α/β itself is inactivated by Akt-mediated phosphorylation (Ser21: α, Ser9: β) (48,49).

---

**Figure 2.** Analysis of proliferative and angiogenesis indices and chemopreventive efficacy of 4-methylumbelliferone (4-MU). A and B) Ki67 positive nuclei (A) and microvessels per high power field (B) in prostate specimens from the vehicle and treatment groups (as shown in Figure 1D) were counted in five independent fields (400X magnification). Data are means, and error bars represent standard deviation. C) In the treatment groups described under Figure 1, 4-MU treatment was suspended after 28 weeks and 50% of the mice were monitored for tumor growth. Prostate and seminal vesicles (P+SV) weights were measured in the vehicle at about 35 weeks (n = 7), and in the 8–28 (n = 4), 12–28 (n = 3), and 22–28 (n = 4) week treatment groups after 52, 44, and 34 weeks, respectively. Data are means, and error bars represent standard deviation. D) Normalized cluster of differentiation antigen 44 (CD44) and hyaluronan-mediated motility receptor (RHAMM) transcript levels in prostate tissues from vehicle and the 8–28 week treatment group. Data are means of three independent mice in duplicate, and error bars represent standard deviation. 4-MU = 4-methylumbelliferone; CD44 = cluster of differentiation antigen 44; RHAMM = hyaluronan-mediated motility receptor.
In T+ prostate tissues, when compared with the vehicle group, 4-MU treatment (8–28 week group) statistically significantly downregulated CD44, RHAMM, β-catenin, and Zeb2 transcript levels, but increased E-cadherin mRNA levels (P < .001) (Figures 2D and 3A). However, the differences in Zeb1, Twist, and Snail levels were not statistically significant (P > .05) (Figure 3A). Immunoblot analysis showed a complete loss of phospho (p) Akt (Ser473), pGSK-3α/β and pβ-catenin (Ser552), and a downregulation of CD44, RHAMM, β-catenin, and Zeb2 in all treatment groups; contrarily, pβ-catenin (Thr41/Ser45), E-cadherin, and caspase-3 levels were upregulated (Figure 3B). 4-MU induced similar changes in the transcript and/or protein levels of CD44, RHAMM, pAkt, pGSK-3α/β, cleaved caspase-3, and EMT markers in DU145 cells (Figure 3, C and D).

Targeting of HA Signaling by 4-MU

We have shown that in PCa cells, 4-MU inhibits HA synthesis and downregulates HA signaling including Akt activation (39). In PC3-ML cells, HA addition prevented 4-MU-mediated downregulation of CD44, RHAMM, β-catenin, and Zeb-2 transcript and protein levels and upregulation of E-cadherin expression (Figure 4, A-C). It also prevented 4-MU-induced downregulation of pAkt, pGSK-3α/β, and pβ-catenin (Ser552) and upregulation of pβ-catenin (Thr41/Ser45) (Figure 4C).

When compared with control siRNA transfection, downregulation of both HA receptors in PC3-ML cells by siRNA transfection decreased Zeb-2 (mean [n = 2] = control: 1.61; CD44+RHAMM siRNA: 0.78) and β-catenin (mean [n = 2] = control: 10.9; CD44+RHAMM siRNA: 6.8) transcript levels and increased E-cadherin expression (mean [n = 2] = control: 0.29; CD44+RHAMM siRNA: 1.1) (Figure 4D). Therefore, effects of 4-MU on EMT effectors are caused by abrogation of HA receptor–mediated signaling, subsequent to HA synthesis inhibition.

In the mAkt-transfected PC3-ML cells, which constitutively overexpress mAkt, 4-MU did not downregulate CD44, RHAMM, β-catenin, or Zeb2 transcript levels or upregulate E-cadherin (Figure 5A). Furthermore, 4-MU did not substantially affect the protein levels of these effectors nor their phosphorylated forms (ie, pAkt, pGSK-3α/β, pβ-catenin [Ser552], pβ-catenin [Thr41/Ser45]) (Figure 5B).

Inhibition of PI-3K Activation by 4-MU

Because 4-MU inhibits Akt activation, we determined whether 4-MU downregulated PI-3K activity. In PC3-ML cells treated

---

**Figure 3.** Effect of 4-methylumbelliferone (4-MU) on the expression of hyaluronic acid (HA) receptors, epithelial mesenchymal transition (EMT) markers, and Akt signaling in transgenic adenocarcinoma of the prostate prostate tissues and DU145 cells. A and B) At 28 weeks, prostate tissues from vehicle and 8–28 week treatment groups, were subjected to reverse transcription quantitative polymerase chain reaction (RT-q-PCR) (A). Data are means of three independent mice in duplicate, and error bars represent standard deviation. B) Immunoblot analysis for HA receptors, epithelial mesenchymal transition markers, and/or proteins involved in Akt signaling (Akt, p-Akt, GSK-3α/β, pGSK-3α/β, pβ-catenin) and apoptosis. Actin was used as a loading control. C and D) Treatment of DU145 cells with 4-MU for 48 hours. C) RT-q-PCR; data are mean of two measurements. D) Immunoblot analysis. Actin was used as a loading control.
with 4-MU, PI-3K activity was downregulated by approximately 95% (mean, SD = control: 0.43, 0.033; 4-MU: 0.022, 0.005; P < .0001) (Figure 5C). Treatment of PC3-ML cells with LY29400, a PI-3K inhibitor, and 4-MU synergistically inhibited cell growth (mean cell no. [x 10^4], SD = control: 8.3, 0.49; 4-MU+LY29400: 1.0, 0.23; combination index: 0.061). This growth inhibition was because of increased apoptosis (mean [OD], SD = control: 0.074, 0.001; 4-MU+LY29400: 0.45, 0.006; P < .001) (Figure 5D). We have previously shown that PI-3K forms a complex with CD44 (10). PLA followed by confocal microscopy showed that in PC3-ML cells CD44 and PI-3K were present in the same microdomains (proximity < 40 nm); however, the complex formation was inhibited more than 90% in 4-MU treated cells (Figure 6A).

**Efficacy of 4-MU in Xenograft and Bone Metastasis Models**

In the DU145 subcutaneous xenograft model, daily gavage of 4-MU inhibited tumor growth by 85% to 90% regardless of whether the treatment began at the time of tumor cell injection (day 0) or when the tumors became palpable on day 8 (tumor volume [mm^3] mean, SD = vehicle: 130, 25; 4-MU (day 0): 7.5, 3.6; 4-MU (day 8): 20.9, 12.2; P = .002) (Figure 6B). Furthermore, 70% of animals did not form tumors at the endpoint (ie, 46 days), although the treatment was terminated on day 28.

In the PC3-ML/Luc intracardiac bone metastasis model, vehicle-treated mice developed skeletal metastasis in the jaw, pelvis, femur, and spinal cord within seven weeks (Figure 6C). However, regardless of whether 4-MU treatment started five days before (Groups 1 and 2) or at the time of (Group 3) PC3-ML/Luc cell injection, no animals in any treatment group developed skeletal metastasis even after 14 weeks, although the treatment was terminated at seven weeks (Figure 6C).

**Comparison of 4-MU Products**

We compared the efficacy of 4-MU with Cholspamin Forte—4-MU sold as an over-the-counter dietary supplement in Europe. At equivalent concentrations, cytotoxic activities of Cholspamin Forte and 4-MU were comparable (Supplementary Figure 1D, available online).

**Effect of 4-MU on Androgen-Dependent Growth**

It has been reported that at 1 µM concentration 4-MU inhibits glucuronidation of androgen and, therefore, increases the androgen-dependent growth of LNCaP cells (50). In a similar study design, dihydrotestosterone (DHT; 10nM) alone increased the growth of LNCaP cells by 174% (mean [cell no. [x 10^4]], SD = control 5.4, 1.0; DHT: 9.4, 1.1; P < .001). However, in the present study, 4-MU (1 µM) did not affect LNCaP cell growth either alone or in the presence of DHT (P > .05) (Supplementary Figure 1F, available online).
Our study demonstrates that 4-MU, which is consumed for improving liver health, has clinically significant chemopreventive and therapeutic efficacy against PCa development, growth, and metastasis, without toxicity. Furthermore, the study delineates the mechanism that dictates this efficacy.

Because of serum PSA testing, there is increased frequency in the detection of low-volume, low-risk PCa (stage T1C). In these patients a nontoxic dietary supplement with potent chemopreventive properties can delay or avoid prostatectomy/radiation therapy by inhibiting disease progression (51). In the stage-specific treatment design, for the 8 to 28 and 12 to 28 week treatment groups, 4-MU treatment began at the PIN and well-differentiated adenocarcinoma stages, respectively; these stages represent low-volume, low-risk PCa. In these groups 4-MU treatment inhibited PCa development for up to one year. This suggests that 4-MU has strong chemopreventive and therapeutic efficacies, and furthermore, the earlier the treatment begins the better the protective effect. Even when treatment began at the poorly differentiated carcinoma stage (22–28 week group), 4-MU not only inhibited PCa growth but also restored the prostate acini architecture. The observed potent antimetastatic effects of 4-MU, regardless of the stage at which the treatment commenced, suggest that 4-MU is effective against advanced PCa.

Although the TRAMP model is probably the most aggressive transgenic model of PCa, in late stages it expresses neuroendocrine phenotype markers (42,43). Furthermore, Akt activation promotes neuroendocrine differentiation (52). 4-MU treatment did not statistically significantly alter the expression of neuroendocrine markers and of SV40 T-antigen, suggesting that the antitumor and antimetastatic effects of 4-MU are unrelated to the transgene expression and the neuroendocrine phenotype. Although PCa frequently metastasizes to bone, transgenic PCa models rarely develop bone metastasis (53). However, the PC3-ML/Luc+ intracardiac injection model reliably develops bony metastasis (54). In this model, regardless of whether the treatment was started a few days before or on the day of tumor cell injection, 4-MU abrogated/prevented skeletal metastasis. 4-MU has also shown efficacy against bone metastasis in a breast cancer model (55). Therefore, daily intake of 4-MU could potentially prevent bone metastasis in high-risk patients and have efficacy against metastatic PCa.
Doses of 4-MU (200–450 mg/kg) used in our study are equivalent to the dose in humans (1.5–2 g/day) as a dietary supplement. Although at these effective doses 4-MU has no detectable toxicity, they are higher than the doses of most anticancer drugs. Targeting the HA pathway with other agents has met with limited success. D-mannose inhibits HA synthesis at high concentrations (IC₅₀ = 20 mM [56]); however, its antitumor activity has not been evaluated. A Curcumin analogue (hylin) inhibits multidrug resistance protein 5–mediated export of HA in fibroblasts (IC₅₀ ~ 5 µM [57]). However, the antitumor activity of this analog has not been evaluated. We have previously shown that sulfated HA, a hyaluronidase inhibitor, displays antitumor activity by abrogating HA signaling. However, its oral bioavailability and activity in transgenic or experimental metastasis models have not been evaluated (10). Pharmacokinetic studies in rodents have shown that 4-MU is biotransformed into sulfated and glucuronidated derivatives in the liver, kidney, and intestine. While 4-MU and its sulfated derivative undergo futile cycling, glucuronidation is the terminal transformation; glucuronidated derivatives of 4-MU are cleared by urine excretion (58–62). Therefore, based on the favorable toxicity profile and high efficacy in three different models of PCa, our study shows that 4-MU can be potentially translated to clinical trials (23–27).

Inhibition of bone metastasis by 4-MU in the PC3-ML model is notable because these cells are PTEN negative (63). Loss of PTEN is associated with higher Gleason grade and PCa recurrence, as it increases Akt activation (64). We have previously shown that 4-MU has potent cytotoxic and anti-invasive activities in PCa cells, regardless of their androgen sensitivity, or androgen receptor, p53, Rb, and/or PTEN status (39). Furthermore, 4-MU also does not alter androgen receptor expression (unpublished results). Contrary to a study that reported that as a “UDP-glucuronate scavenger” at 1 µM concentration 4-MU competitively inhibits glucuronidation of androgen and increases androgen-dependent growth of LNCaP cells, in our study 4-MU did not display this effect (50). Based on the high Km values of UDP-glucuronosyltransferases (0.1–0.9 mM) it is intriguing how 4-MU would competitively inhibit the

Figure 6. Efficacy of 4-methylumbelliferone (4-MU) in prostate cancer (PCa) xenograft models and its mechanism of action. A) Proximal ligation assay (PLA) for phosphoinositide 3-kinase (PI-3K) and cluster of differentiation antigen 44 (CD44). PC3-ML cells treated with 4-MU (0, 0.4 mM) for 24 hours were subjected to PLA using anti-PI-3K (α, p85 subunit) and anti-CD44 antibodies. Confocal microscopy images at ×630 magnification are shown. a) IgG control. b and c) Cells treated with 0 and 0.4 mM 4-MU, respectively. Red dots indicate the presence of the CD44 and PI-3K complex. 630X magnification. B) DU145 xenograft. Athymic mice implanted subcutaneously with DU145 cells were gavaged daily with vehicle or 4-MU (450 mg/kg), starting on day 0 or day 8, for 28 days. Mice in day 0 group were monitored until day 46, although the treatment was terminated at day 28. Data are means of seven mice per group, and error bars represent standard deviation. C) PC3-ML skeletal metastasis model. Athymic mice were gavaged with vehicle or 4-MU (200 mg/kg: Group 1; 450 mg/kg: Group 2) starting five days before the implantation of PC3-ML/Luc+ cells by intracardiac route or with 450 mg/kg 4-MU on the day of tumor cell injection (Group 3). Vehicle: images of mice at week 7. Groups 1–3: Images of mice at week 14 (note: the treatment was terminated at week 7). D) Proposed model for the mechanism of action for 4-MU. 4-MU = 4-methylumbelliferone; CD44 = cluster of differentiation antigen 44; RHAMM = hyaluronan-mediated motility receptor.
glucuronidation of substances including androgen at 1 μM concentration. Based on the present and published studies, inhibition of HA synthesis appears to be the major mechanism for the antitumor activity of 4-MU (22-29, 39).

Based on the mechanistic findings of our study, we propose that HA present in the tumor-associated matrix binds HA receptors (CD44, RHAMM) and promotes a complex formation between HA receptors and PI-3K at the plasma membrane. This complex activates PI-3K, which in turn activates Akt and downstream signaling—GSK-3 α/β inactivation, β-catenin stabilization/activation, β-catenin and Zeb-2 upregulation, E-cadherin downregulation, and upregulation of HA receptors (feedback loop). This intracellular signaling promotes proliferation, motility/invasion, EMT, metastasis, and angiogenesis (Figure 6D). By inhibiting HA synthesis, 4-MU blocks the first step in this pathway, causing inhibition of PI-3K/Akt signaling and EMT reversal (Figure 6D). β-catenin is an important mediator of Wnt signaling and consistent with the decreased β-catenin levels; 4-MU also downregulated cyclin D1 levels, a β-catenin/Wnt pathway effector. However, 4-MU plausibly does not directly affect the Wnt pathway, because it did not alter dishevelled levels/status (unpublished results [65]). HA rescue and mAkt overexpression experiments further emphasize the presence of a feedback loop between Akt activation and HA receptors. Therefore, by inhibiting HA synthesis, 4-MU displays potent chemopreventive and therapeutic efficacy against Pca development, growth, metastasis, and angiogenesis in preclinical models.

In summary, our study demonstrates that the nontoxic dietary supplement 4-MU displays potent chemopreventive and therapeutic efficacy against localized and metastatic Pca in preclinical models by targeting HA signaling. A limitation of our study is that the efficacy of 4-MU was evaluated in preclinical models only. Although, the doses of 4-MU used in the mouse models were comparable with those in human use as a dietary supplement and 4-MU was found to be nontoxic, phase I clinical trials are warranted for dose optimization and evaluation of toxicity (if any) at the therapeutic dose.

Funding
Grant support: 1R21CA184018-01 (VBL); R01 CA 123063-04 (VBL); National Cancer Institute/National Institutes of Health 1R01CA176691-02 (VBL); R01 CA72821-15 (VBL).

Notes
We thank Dr. Michael Henry, University of Iowa, for the PQQXIN-luciferase construct and Dr. Barbara Foster, Roswell Park Cancer Institute, for the K67 immunohistochemistry protocol. We are grateful to Dr. Suzanne Forry, National Cancer Institute, for helpful suggestions during the course of this study. We thank Mr. Bernard J. Wasserlauf from the In Vivo Imaging Core, Sylvester Comprehensive Cancer Center, University of Miami for providing assistance in live animal imaging.

References


